Bactericidal Activity of Mouse α -Defensin Cryptdin-4 Predominantly Affects Noncommensal Bacteria

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Key Words

Cryptdin-4 • Bacteria, commensal, noncommensal • Disulfide bonds

Abstract

Mouse Paneth cell α -defensins, termed cryptdins, are secreted into the intestinal lumen, have microbicidal activity, and contribute to intestinal innate immunity. Among them, cryptdin-4 (Crp4) has the most potent microbicidal activity. In the intestinal lumen, commensal bacteria colonize and elicit beneficial effects in the host. However, the effects of Crp4 against commensal bacteria are poorly understood. Thus, we investigated the bactericidal activities of Crp4 against commensal bacteria compared to noncommensal bacteria. Oxidized Crp4 showed only minimal or no bactericidal activity against 8 out of 12 commensal bacterial species, including *Bifidobacterium bifidum* and *Lactobacillus casei*. We further addressed a role of the conserved disulfide bonds of Crp4 by analyzing reduced Crp4 (r-Crp4). r-Crp4 demonstrated significantly greater bactericidal activities against 7 of 12 commensal bacteria than did oxidized Crp4. Oxidized Crp4 and r-Crp4 elicited equivalently potent bactericidal activities against 11 of the 11 noncommensal bacteria tested, such as *Salmonella enterica* serovar Typhimurium,

and against 5 of 12 commensal bacteria. Furthermore, when r-Crp4 was exposed to a processing enzyme of cryptdins, i.e. MMP-7, r-Crp4 was degraded and the bactericidal activities disappeared. These findings suggest that Crp4 has selective bactericidal activities against intestinal microbiota and that the activities are dependent on the disulfide bonds.

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Introduction

Innate immunity functions as the front line of host defense in plants, invertebrates, and mammals. Antimicrobial peptides (AMPs) are one of the major effectors of innate immunity [1–3]. In the small intestine, AMP α -defensins are expressed in the granules of Paneth cells and are secreted into the lumen of intestinal crypts in response to bacterial stimuli [4]. The secreted α -defensins elicit potent bactericidal activity and contribute to innate immunity in the small intestine [5, 6]. Mouse α -defensins, termed cryptdins (Crps), are activated in vivo in Paneth cell granules through the processing of pro-Crps with the proteolytic enzyme matrix metalloproteinase-7 (MMP-7; matrilysin) [7, 8]. MMP-7 cleaves pro-Crps at 3 cleavage sites, and 1 of these sites is the N-terminus or

near the N-terminus of mature Crps [9]. This processing is an essential event for the production of mature, functional Crps [7–9]. MMP-7-deficient mice that lack the active form of Crps are significantly more susceptible to orally administered *Salmonella enterica* serovar Typhimurium than are wild-type mice [7]. These results also show the pivotal role of AMPs in innate immunity.

In the gastrointestinal tract, a highly complex microbial ecosystem is constructed by colonizing microbes. Hosts and microbiota have coevolved in ways that have mutually beneficial effects [10]. These include host development [11], nutritional absorption [12], and the functional development of the immune systems [13]. Recent studies have shown that well-balanced cross talk between the host and commensal bacteria is important [14] as imbalances in the relationships result in inflammation [15] and cancer [16]. In the mouse small intestine, the expression of more than 20 cryptdin genes and cryptdin-related sequence genes has been reported [17–20], and these peptides are secreted into the intestinal lumen where commensal bacteria reside [21]. Among Crps, cryptdin-1 to cryptdin-6 (Crp1 to Crp6) are characterized at the peptide level. Although the amino acid sequence identities of mature regions of Crp1 to Crp3 and Crp6 are more than 90%, those of Crp4 and Crp5 with Crp1 are 42 and 54%, respectively [18]. In particular, Crp4 has several features that distinguish it from other Crps. For example, the Crp4 polypeptide chain uniquely lacks 3 amino acids between the fourth and fifth cysteine residue positions [18] and the unique repeated element in the region upstream of the gene transcriptional start site [22]. Crp4 also has the most potent in vitro bactericidal activity of known mouse Paneth cell α -defensins [18], suggesting that Crp4 may play a pivotal role in intestinal innate immunity. However, the interaction of Crp4 and small intestinal microbiota is poorly understood. Thus, to clarify the effects of Crp4 on commensal bacteria, we investigated the bactericidal activities of Crp4 against commensal bacteria compared to noncommensal bacteria. We further tested the bactericidal activities of Crp1 in addition to Crp4.

Crp4 is a highly cationic peptide and is generally believed to permeabilize the bacterial plasma membrane through electrostatic interaction with negatively charged bacterial phospholipids followed by the insertion of hydrophobic side chains [23]. This conclusion is supported by reports which reveal that positively charged Arg residues of Crp4 are critical for its bactericidal activity [24]. However, the precise mechanisms of its bactericidal activity are not known. α -Defensins including Crp4 are characterized by invariant disulfide bonds arranged be-

tween Cys¹–Cys⁶, Cys²–Cys⁴, and Cys³–Cys⁵ [3]. The pairings of 3 disulfide bonds are conserved in all species which express these peptides. Previously, the bactericidal activities of the Crp4 mutants in which Cys residues were substituted to Ala residues were analyzed by Maemoto et al. [25], who showed that disulfide bond-null mutants had equivalent or greater bactericidal activity compared to native Crp4 [25]. However, the effects of the disulfide bonds in native Crp4 on the bactericidal activity against commensal bacteria remain unknown. Therefore, we further addressed a role of the disulfide bonds in the bactericidal activity of Crp4 using reduced Crp4 (r-Crp4) which did not contain disulfide bonds.

Crp4 is processed by MMP-7 in the granules of Paneth cells. It has been reported that the disulfide bond-null mutant of Crp4 is degraded by MMP-7 [25]. This result indicates that the disulfide bonds of Crp4 determine the proteolytic resistance to MMP-7. Therefore, to elucidate the effect of reduction on the processing of r-Crp4, we also investigated the susceptibility of r-Crp4 to MMP-7 and the effects of MMP-7 on the bactericidal activity of the processed r-Crp4.

Materials and Methods

Preparation of Oxidized Crps and r-Crps

Three pairs of disulfide bonds were introduced into Crp4 (Sigma Genosys, St. Louis, Mo., USA, or Medical and Biological Laboratories Co., Ltd., Nagoya, Japan) by air oxidation as described previously [25]. Crp4 was then purified to homogeneity using reverse-phase high-performance liquid chromatography (RP-HPLC). The Crp4 with 3 pairs of disulfide bonds, defined as oxidized Crp4, was purified by a C-18 column (SepaxHP-C18, 4.6 × 150 mm, 5 μ m; Sepax Technologies, Inc., Newark, Del., USA) in 0.1% trifluoroacetic acid with an 18–36% acetonitrile gradient developed over 30 min at 1 ml/min (online supplementary figure S1a, www.karger.com/doi/10.1159/000322037). Oxidized Crp4 was obtained after the final lyophilization and stored at –30°C until use.

r-Crp4, in which the disulfide bonds were entirely reduced, was prepared. Oxidized Crp4 was dissolved in 500 mM dithiothreitol (DTT) and left to stand at 4°C overnight. The reaction mixture was then applied to a C-18 column and r-Crp4 was purified by RP-HPLC under the same conditions used in the purification of oxidized Crp4 (online suppl. fig. S1b). Because the retention times of DTT and r-Crp4 were very different, we concluded that DTT was completely removed from r-Crp4 in the purification process. Thus, the r-Crp4 used in the assay did not contain DTT, and we confirmed that DTT did not affect the bactericidal assay. r-Crp4 was obtained after the final lyophilization and stored at –30°C until use. Oxidized Crp1 and r-Crp1 were also prepared by the same respective methods.

Evaluation of Disulfide Bond Formation

Disulfide bond formation was evaluated by acid-urea PAGE (AU-PAGE) [26], MALDI-TOF MS, and RP-HPLC. In the AU-PAGE analysis, samples (1.0 µg) of oxidized Crp4 and r-Crp4 were dissolved in 5% acetic acid and electrophoresed on 12.5% acrylamide gel containing 5% acetic acid and 5 M urea at 150 V [26]. Thereafter, the gel was stained with Coomassie brilliant blue R-250. The molecular masses of the peptides were determined by MALDI-TOF MS (Voyager-DE PRO; Applied Biosystems, Carlsbad, Calif., USA). Oxidized Crp4 and r-Crp4 were analyzed by RP-HPLC using a C-18 column under the same conditions used in the purification of oxidized Crp4.

Bacterial Strains and Culture Conditions

As some of the most common commensal bacteria in the small intestine [27–30], *Bifidobacterium bifidum* ATCC 11863 (*B. bifidum*), *Bifidobacterium breve* JCM 1192 (*B. breve*), *Bifidobacterium longum* ATCC 15707 (*B. longum*), *Lactobacillus acidophilus* ATCC 314 (*L. acidophilus*), *Lactobacillus casei* ATCC 393 (*L. casei*), *Lactobacillus johnsonii* JCM 2012 (*L. johnsonii*), *Bacteroides fragilis* JCM 11019 (*B. fragilis*), *Bacteroides ovatus* JCM 5824 (*B. ovatus*), *Bacteroides thetaiotaomicron* JCM 5827 (*B. thetaiotaomicron*), *Bacteroides vulgatus* JCM 5826 (*B. vulgatus*), *Enterococcus faecalis* JCM 5803 (*E. faecalis*), and *Enterococcus faecium* JCM 5804 (*E. faecium*) were used. As examples of noncommensal bacteria in the small intestine, wild-type *S. enterica* serovar Typhimurium ATCC 14028 (*S. enterica* serovar Typhimurium), a defensin-sensitive strain of *S. enterica* serovar Typhimurium *phoP*– (*S. enterica* serovar Typhimurium *phoP*–) [31], *Escherichia coli* ML35 ATCC 43827 (*E. coli*), *Staphylococcus aureus* ATCC 27217 (*S. aureus*), *Listeria monocytogenes* JCM 7671 (*L. monocytogenes*), *Klebsiella oxytoca* JCM 1665 (*K. oxytoca*), *Klebsiella pneumoniae* JCM 1662 (*K. pneumoniae*), *Proteus vulgaris* JCM 20013 (*P. vulgaris*), *Yersinia enterocolitica* JCM 7577 (*Y. enterocolitica*), *Campylobacter coli* JCM 2529 (*C. coli*), and *Campylobacter jejuni* JCM 2013 (*C. jejuni*) were used. Bacteria were cultured in the following media: *B. bifidum*, reinforced clostridial medium (RCM) supplemented with 2% (w/v) skim milk; *B. breve* and *B. longum*, RCM; *Lactobacillus* sp., de Man, Rogosa, and Sharpe (MRS) broth; *Bacteroides* sp., GAM broth (Nissui Seiyaku Co., Ltd., Tokyo, Japan); *Enterococcus* sp., brain heart infusion (BHI); noncommensal bacteria except for *C. coli* and *C. jejuni*, tryptic soy broth, and *C. coli* and *C. jejuni*, GAM broth. Commensal bacteria were grown in anaerobic conditions using the Anaero Pack system (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan) at 37°C. Noncommensal bacteria, except for *C. coli* and *C. jejuni*, were grown in a shaking incubator at 37°C with shaking at 180 rpm. *C. coli* and *C. jejuni* were grown in microaerophilic conditions using the Anaero Pack system.

Bactericidal Peptide Assay

Exponential-phase bacteria cultured at 37°C were deposited by centrifugation at 9,300 g at 4°C for 5 min. Bacteria, except for *B. vulgatus*, were washed twice and resuspended in Milli-Q water; *B. vulgatus* was washed twice and resuspended in PBS diluted 1:4 with Milli-Q water. The OD₆₂₀ was measured to determine bacterial cell numbers. Twenty microliters of samples containing 1,000 colony-forming units (CFU) per aliquot were mixed with equal volumes of oxidized Crp4, r-Crp4, oxidized Crp1, or r-Crp1 to final concentrations ranging from 0.027 to 1.35 µM. The mixtures

were incubated for 1 h at 37°C. The incubated samples were plated on RCM agar plates for *Bifidobacterium* sp., on MRS agar plates for *Lactobacillus* sp., on GAM agar plates for *Bacteroides* sp., on BHI agar plates for *Enterococcus* sp., and on tryptic soy agar (TSA) plates for noncommensal bacteria. The plates were then incubated in anaerobic conditions at 37°C for commensal bacteria or at 37°C for noncommensal bacteria. Bacterial survival rates were determined from surviving colonies relative to peptide-unexposed controls (online suppl. fig. S2). The bacterial cell viability of the peptide-unexposed controls was not changed during bactericidal peptide assay (data not shown).

Bactericidal Peptide Assay against a Bacterial Mixture

Exponential phase *S. enterica* serovar Typhimurium, *L. casei*, and *B. thetaiotaomicron* cultured at 37°C were washed with Milli-Q water, and each bacteria population was adjusted to 150 CFU in 20 µl. Subsequently, each bacterial solution of 20 µl was mixed, and the mixture was incubated with 60 µl of oxidized Crp4 or r-Crp4 with a final concentration of 1.35 µM. After incubation for 1 h at 37°C, the bacterial mixtures were separated and grown on TSA plates in aerobic conditions and on MRS and GAM plates in anaerobic conditions using the Anaero Pack system. More than 28 colonies were picked randomly from each plate as being representative of the total colonies. Subsequently, the genomic sequences of the conserved region in 16S rRNA among 3 species of bacteria were amplified by colony-direct polymerase chain reaction (PCR) using Blend Taq (TOYOBO, Tokyo, Japan). The forward primer (5'-GTTGG TGAGG TAACG GCTCA CCAA-3') was paired with the reverse primer (5'-TGACG GGCGG TGTGT ACAAG GC-3'). The PCR products from *S. enterica* serovar Typhimurium, *L. casei*, and *B. thetaiotaomicron* were digested at only 1 site by *Bam*H I, *Bgl* II, and *Spe* I, respectively. Therefore, after the digestion of the PCR products by these 3 enzymes, each bacterium was distinguished by the resulting patterns visualized by agarose electrophoresis. The relative bacterial distribution was shown based on chosen representative colonies, and the actual colony numbers of each bacterium were estimated by multiplying the relative bacterial distribution by the total colony numbers. Because each plate is appropriate for each bacterium, the numbers of *S. enterica* serovar Typhimurium, *L. casei*, and *B. thetaiotaomicron* were estimated from colonies grown on TSA, MRS, and GAM plates, respectively.

Antimicrobial Assay with Membrane Potential-Sensitive Dye

Exponential-phase bacteria were incubated in Milli-Q water at 37°C with oxidized Crp4 or r-Crp4 (1.35 µM) for 1 h. The suspensions were then incubated for 10 min with 1 µg/ml of the membrane potential-sensitive fluorophore, i.e. bis-(1,3-dibutylbarbituric acid) trimethine oxonol [DiBAC₄(3)] (Invitrogen, Carlsbad, Calif., USA), as described previously [32]. The suspensions were centrifuged for 5 min at 9,300 g, and the bacterial pellets were resuspended in 1 ml PBS(–). Each bacterial sample was analyzed on a JSAN desktop cell sorter (Bay Bioscience, Kobe, Japan). The median fluorescence intensity ratio was obtained by dividing the median fluorescence intensity of the peptide-treated sample by the median fluorescence intensity of the nontreated sample.

Cleavage of r-Crp4 with MMP-7 *in vitro*

Samples (1.0 μ g) of oxidized Crp4 and r-Crp4 were incubated with an activated recombinant human MMP-7 (1.0 μ g) catalytic domain (Calbiochem, La Jolla, Calif., USA) in 10 mM HEPES pH 7.4, 150 mM NaCl, and 5 mM CaCl_2 for 18–24 h at 37°C [9, 25]. The digested samples were analyzed by Tris-Tricine SDS-PAGE, N-terminal peptide sequencing, and MALDI-TOF MS. For the N-terminal peptide sequencing by Edman degradation, digested r-Crp4 was resolved by RP-HPLC under the same conditions used

in the purification of oxidized Crp4. Samples (50 pmol) of digested r-Crp4 were subjected to 5 cycles of N-terminal peptide sequencing at the Creative Research Initiative ‘Sousei’, Hokkaido University. To determine biological activity, the digested samples were applied to bactericidal peptide assays as described above.

Results

Oxidized Crp4 Shows Bactericidal Activities against Noncommensal Bacteria but Little or No Bactericidal Activity against Some Commensal Bacteria

Throughout the present study, we used oxidized Crp4 containing 3 pairs of disulfide bonds and r-Crp4 obtained by the reduction of oxidized Crp4. As shown in the results of AU-PAGE (fig. 1), MALDI-TOF MS (fig. 2a), and RP-HPLC (online suppl. fig. S1a), oxidized Crp4 was homogeneous. Oxidized Crp4 showed a lower molecular weight than r-Crp4 by 6 Da (fig. 2), consistent with the oxidation of 6 Cys residues in the formation of disulfide pairings. Furthermore, oxidized Crp4 had bactericidal activity against 2 strains of *S. enterica* serovar Typhimurium, as well as against *E. coli* and *S. aureus* (fig. 3). Hence, the prepared oxidized Crp4 was biologically active and contained 3 pairs of disulfide bonds. r-Crp4 showed a reduced migration relative to oxidized Crp4 in AU-PAGE (fig. 1). This corresponds to the results of AU-PAGE for oxidized Crp4 versus Cys-to-Ala-substituted Crp4, in which the disulfide-null mutant showed a reduced migration compared to oxidized Crp4 [25]. MALDI-TOF MS of

Fig. 1. AU-PAGE analysis of oxidized Crp4 and r-Crp4. Samples (1.0 μ g) of oxidized Crp4 and r-Crp4 were resolved by AU-PAGE (12.5% acrylamide gel containing 5% acetic acid and 5 M urea) and then stained with Coomassie brilliant blue R-250.

Fig. 2. MALDI-TOF MS analysis of oxidized Crp4 and r-Crp4. The molecular weights of oxidized Crp4 (a) and r-Crp4 (b) analyzed by MALDI-TOF MS are shown.

Fig. 3. Bactericidal activities of oxidized Crp4 and r-Crp4 against noncommensal bacteria. The survival rates of *S. enterica* serovar Typhimurium (a), *S. enterica* serovar Typhimurium *phoP*⁻ (b), *E. coli* ML35 (c), *S. aureus* (d), *L. monocytogenes* (e), *K. oxytoca* (f), *K. pneumoniae* (g), *P. vulgaris* (h), *Y. enterocolitica* (i), *C. coli* (j), and *C. jejuni* (k) exposed to oxidized Crp4 (●) or r-Crp4 (□) at 0.027, 0.054, 0.135, 0.27, and 1.35 μ M are shown. Data are expressed as means $\pm$ SE; n = 6 for a–d; n = 3 for e–k. Experiments were performed in triplicate.

Fig. 4. Bactericidal activities of oxidized Crp4 and r-Crp4 against commensal bacteria. The survival rates of *B. bifidum* (a), *B. breve* (b), *B. longum* (c), *L. acidophilus* (d), *L. casei* (e), *L. johnsonii* (f), *B. fragilis* (g), *B. ovatus* (h), *B. thetaiotaomicron* (i), *B. vulgatus* (j), *E. faecalis* (k), and *E. faecium* (l) exposed to oxidized Crp4 (●) or r-Crp4 (□) at 0.027, 0.054, 0.135, 0.27, and 1.35 μM are shown. Data are expressed as means $\pm$ SE; $n = 6$ for a, c–e, i, and j; $n = 3$ for b, f–h, k, and l. Experiments were performed in triplicate. * $p < 0.01$ by Student's t test.

Fig. 5. Statistical analysis of bactericidal activities. The survival rates of 12 commensal bacteria and 11 noncommensal bacteria (Material and Methods) exposed to oxidized Crp4 or r-Crp4 at 1.35 μ M are shown. Each point represents a survival rate of each bacterium. The medians are expressed as horizontal lines; the data were evaluated for statistical significance using the Mann-Whitney U test where differences at values of * $p < 0.05$ were considered to be significant. NS = Not significant.

r-Crp4 showed a major peak (fig. 2b) and RP-HPLC of r-Crp4 showed a single peak (online suppl. fig. S1b). From these results, we judged that r-Crp4 was homogeneous and that the 3 pairs of disulfide bonds were reduced. The reduction state of r-Crp4 was assured by MALDI-TOF MS and AU-PAGE prior to use and after incubation in bactericidal assays. Although r-Crp4 showed secondary bands in AU-PAGE suggestive of disulfide bond formation, some Cys residues may tend to form disulfide bonds in AU-PAGE since an excess concentration of DTT is needed for the complete reduction of Crp4.

The bactericidal activities of oxidized Crp4 against small intestinal commensal and noncommensal bacteria were examined. Oxidized Crp4 killed 11 of 11 noncommensal bacteria and 4 of 12 commensal bacteria, i.e. *B. longum*, *B. vulgatus*, *E. faecalis*, and *E. faecium*, in a dose-dependent manner (fig. 3, 4c, j–l). In contrast, oxidized Crp4 showed little or no bactericidal activities against 8 out of 12 commensal bacteria, i.e. *B. bifidum*,

Fig. 6. Bactericidal activities of oxidized Crp4 and r-Crp4 against the mixture of commensal and noncommensal bacteria. The bacterial mixture of exponentially growing *S. enterica* serovar Typhimurium as noncommensal bacteria and *L. casei* and *B. thetaiotaomicron* as commensal bacteria was exposed to oxidized Crp4 or r-Crp4 at 1.35 μ M. The surviving bacteria were counted as CFU.

B. breve, *L. acidophilus*, *L. casei*, *L. johnsonii*, *B. fragilis*, *B. ovatus*, and *B. thetaiotaomicron* at 1.35 μ M (fig. 4a, b, d–i). These activities did not change at a 2.7 μ M peptide concentration (data not shown). Thus, oxidized Crp4 had only a minimal effect or no effect on the survival of 8 out of the 12 species of commensal bacteria tested. In contrast, oxidized Crp4 had potent bactericidal activities against 11 of the 11 noncommensal bacterial species tested, but it was only active against 4 of the 12 species of commensal bacteria. The statistical analysis of the bactericidal activities of oxidized Crp4 against commensal bacteria and noncommensal bacteria revealed that oxidized Crp4 showed significantly greater bactericidal activities against noncommensal bacteria than against commensals at 1.35 μ M (fig. 5; commensal bacteria vs. noncommensal bacteria exposed to oxidized Crp4).

In the intestinal lumen, a wide variety of bacteria are able to colonize. To further test the selective activity of oxidized Crp4, a mixture of commensal and noncommensal bacteria was exposed to oxidized Crp4. Oxidized Crp4 selectively killed *S. enterica* serovar Typhimurium, while the viability of the other commensal bacteria, i.e. *L. casei* and *B. thetaiotaomicron*, in the mixture was retained (fig. 6; nontreated and oxidized Crp4-treated). This result supports the selective activities of Crp4.

To determine whether this selective activity is specific to oxidized Crp4, we analyzed the bactericidal activities of oxidized Crp1, the most abundant Crp, against 6 non-

Fig. 7. Antimicrobial assay with membrane-sensitive dye. The median fluorescence intensity ratios (MFIR) of commensal bacteria (a) and noncommensal bacteria (b) exposed to oxidized Crp4 (■) or r-Crp4 (□) are shown. Data are expressed as means $\pm$ SE. Experiments were performed in triplicate, and the results were evaluated for statistical significance by Student's t test. * $p < 0.05$ was considered to be statistically significant. NS = Not significant.

commensal bacteria, i.e. *S. enterica* serovar Typhimurium, *S. aureus*, *E. coli*, *L. monocytogenes*, *K. oxytoca*, and *P. vulgaris*, as well as against 6 commensal bacteria, i.e. *B. bifidum*, *B. longum*, *L. casei*, *L. johnsonii*, *B. fragilis*, and *B. thetaiotaomicron*. Oxidized Crp1 showed potent bactericidal activities against 6 of 6 noncommensal bacteria and against 1 of 6 commensal bacteria, i.e. *B. longum* (online suppl. table S1). However, Crp1 had little or no effect on 5 of 6 commensal bacteria (online suppl. table S1). Thus, oxidized Crp1 also had predominant bactericidal activities against noncommensal bacteria, as did oxidized Crp4.

r-Crp4 Has Bactericidal Activities against both Commensal and Noncommensal Bacteria

Since disulfide bond pairings in α -defensins are conserved in all species which express these peptides, the bactericidal activities of r-Crp4 were analyzed to elucidate the effects of the conserved disulfide bonds on its bactericidal activity. r-Crp4 showed equivalent bactericidal activities against all noncommensal bacteria but only against 5 of 12 commensal bacterial species, i.e. *B. longum*, *L. acidophilus*, *B. vulgatus*, *E. faecalis*, and *E. faecium*, in comparison with oxidized Crp4 (fig. 3, 4c, d, j–l). These data indicated that the bactericidal activities of Crp4 against these bacteria were independent of the ex-

istence of the disulfide bonds. On the other hand, r-Crp4 killed 7 out of 12 commensal bacteria, i.e. *B. bifidum*, *B. breve*, *L. casei*, *L. johnsonii*, *B. fragilis*, *B. ovatus*, and *B. thetaiotaomicron*, in a dose-dependent manner and showed significantly greater bactericidal activities compared to those of oxidized Crp4 (fig. 4a, b, e–i). Therefore, the reduction of the disulfide bonds conferred Crp4 bactericidal activities against *B. bifidum*, *B. breve*, *L. casei*, *L. johnsonii*, *B. fragilis*, *B. ovatus*, and *B. thetaiotaomicron*, but the disulfide bonds had little or no effect on bactericidal activity against all noncommensal bacteria tested and *B. longum*, *L. acidophilus*, *B. vulgatus*, *E. faecalis*, and *E. faecium*. A comparison of the bactericidal activities of oxidized Crp4 and r-Crp4 revealed that r-Crp4 had significantly greater bactericidal activities against commensal bacteria than did oxidized Crp4 (fig. 5; commensal bacteria exposed to oxidized Crp4 vs. r-Crp4). The bactericidal activities of oxidized Crp4 and r-Crp4 were not significantly different against noncommensal bacteria (fig. 5; noncommensal bacteria exposed to oxidized Crp4 vs. r-Crp4).

When the mixture of commensal and noncommensal bacteria was exposed to r-Crp4, r-Crp4 had potent bactericidal activities against both commensals and noncommensals (fig. 6; nontreated and r-Crp4-treated). Therefore, the selective bactericidal activities of Crp4

Fig. 8. Proteolytic degradation assay of oxidized Crp4 and r-Crp4 by MMP-7. **a** Tris-Tricine SDS-PAGE analysis of oxidized Crp4 and r-Crp4 incubated with or without MMP-7. Samples (1.0 μ g) of oxidized Crp4 and r-Crp4 were incubated with or without an MMP-7 (1.0 μ g) catalytic domain. The digested samples were analyzed by Tris-Tricine SDS-PAGE. The positions of bands corresponding to MMP-7 and Crp4 are noted on the right. The bold arrow denotes the position of the MMP-7-digested fragment of r-Crp4. **b** MMP-7 cleavage sites in r-Crp4. The digested r-Crp4 was analyzed by 5 cycles of N-terminal peptide sequencing and MALDI-TOF MS. Cleavage sites are noted by downward arrows ($\downarrow$). The numerals below the r-Crp4 sequence refer to residue positions in reference to the N-terminal Gly of r-Crp4 as residue position number 1.

against certain commensal bacteria were regulated by its disulfide bonds.

Furthermore, the bactericidal activities of r-Crp1 against 6 noncommensal and 6 commensal bacteria were also tested for relative sensitivity to a second mouse α -defensin, oxidized Crp1 and r-Crp1. r-Crp1 showed bactericidal activities equivalent to those of oxidized Crp1 against 6 of 6 noncommensal bacteria and against 1 of 6 commensal bacteria, i.e. *B. longum* (online suppl. table S1), but it demonstrated significantly greater bactericidal activity against 5 of 6 commensal bacteria (online suppl. table S1) compared to oxidized Crp1. Thus, the predominant bactericidal activities of Crp1 against noncommensal bacteria were also affected by its disulfide bonds, as in the case of Crp4.

We next tested the membrane disruption activity of Crp4. It has been reported that a Crp4-permeabilized

bacterial cell membrane and permeabilization correlate with bacterial killing activity [23]. To examine the possible mechanism of the bactericidal activities of Crp4, a depolarization of the membrane potential was detected with membrane potential-sensitive fluoroprobe DiBAC₄(3) [32] after exposure to oxidized Crp4 and r-Crp4. r-Crp4 exhibited significantly greater depolarization than did oxidized Crp4 in 3 of the 3 commensal bacteria tested (fig. 7). On the other hand, the depolarization activities of oxidized Crp4 and r-Crp4 were not significantly different against 2 of 3 species of noncommensal bacteria (fig. 7). Both oxidized Crp4 and r-Crp4 showed a remarkable depolarization against *K. oxytoca* (fig. 7).

Digestion of r-Crp4 by MMP-7 Resulted in the Attenuation of Its Bactericidal Activity

Crps are processed and activated by the processing enzyme, i.e. MMP-7, in vivo in Paneth cell granules. It has been reported that the disulfide bonds of Crp4 are essential for proteolytic resistance to MMP-7 due to the use of the disulfide bond-null mutant of Crp4 [25]. Therefore, to elucidate the susceptibility of r-Crp4 to MMP-7, an assay for proteolytic degradation was conducted. Oxidized Crp4 and r-Crp4 were incubated with or without MMP-7 and then applied to Tris-Tricine SDS-PAGE. r-Crp4 was digested by MMP-7 into smaller fragments, whereas oxidized Crp4 showed complete resistance to MMP-7 (fig. 8a). N-terminal peptide sequencing of the peptide fragment that emerged in the Tris-Tricine SDS-PAGE revealed that the N-terminus was YCRKG. MALDI-TOF MS showed that the peptide fragment had a molecular weight of 2,480 Da. These results indicated that MMP-7 cleaved r-Crp4 at Cys⁴↓Tyr⁵ and Phe²⁵↓Leu²⁶ as shown in figure 8b. Although the peptide fragment appeared to have a molecular weight of $\sim$ 3.5 kDa in figure 8a, because the fragment comprises cationic amino acids at high rates (theoretical pI = 10.3), it might have had a smaller migration in Tris-Tricine SDS-PAGE than that expected based on the molecular weight determined by MALDI-TOF MS. The observed cleavage sites correspond to the previous cleavage of reduced, alkylated pro-Crp4 by MMP-7 at the same sites [9]. Furthermore, the digested peptide fragments were assayed for bactericidal peptide activity to test for biological effects of the degradation. Previously, it was shown that MMP-7 itself had no effect on bactericidal activity [7, 25]. The bactericidal activities of r-Crp4 against commensal and noncommensal bacteria, except for *L. acidophilus*, significantly decreased when r-Crp4 was digested by MMP-7 (table 1). In contrast, the faint bactericidal activity of digested r-Crp4

Table 1. Bactericidal activities of r-Crp4 and MMP-7-digested r-Crp4

Bacteria	Survival rates ^a (%) of bacteria exposed to	
	r-Crp4	MMP-7-digested r-Crp4
<i>S. enterica</i> serovar Typhimurium	1.4 ± 1.2	98.3 ± 9.9 ^b
<i>S. enterica</i> serovar Typhimurium <i>phoP</i> –	3.2 ± 2.7	96.3 ± 12.0 ^b
<i>E. coli</i> ML35	0.0 ± 0.0	118.1 ± 10.9 ^b
<i>S. aureus</i>	0.0 ± 0.0	49.5 ± 13.4 ^b
<i>B. bifidum</i>	13.1 ± 7.3	96.4 ± 10.4 ^b
<i>B. longum</i>	0.2 ± 0.2	87.3 ± 17.0 ^b
<i>L. casei</i>	28.8 ± 7.3	72.4 ± 10.3 ^b
<i>L. acidophilus</i>	53.9 ± 6.3	64.8 ± 0.9 ^c
<i>B. thetaiotaomicron</i>	33.9 ± 5.7	86.5 ± 9.5 ^b
<i>B. vulgatus</i>	13.6 ± 6.6	101.6 ± 8.9 ^b

^a Values are means ± SE for the survival rates of bacteria exposed to r-Crp4 (1.35 μM) and MMP-7-digested r-Crp4 (1.35 μM). n = 6 for r-Crp4; n = 3 for MMP-7-digested r-Crp4.

^b Value is significantly different (p < 0.01) compared to that of bacteria exposed to r-Crp4 as calculated by Student's t test.

^c Not significant.

against *L. acidophilus* remained (table 1). Thus, the degradation of r-Crp4 by MMP-7 attenuated its bactericidal activity against most of the bacteria tested.

Discussion

In this study, the bactericidal activities of oxidized Crp4 against commensal and noncommensal bacteria were analyzed. Consistent with the results of previous studies [18, 25], oxidized Crp4 showed potent bactericidal activities against noncommensal bacteria. In contrast, oxidized Crp4 had only minimal or no bactericidal activity against commensal bacteria, though it did show dose-dependent activity against *B. longum*, *B. vulgatus*, *E. faecalis*, and *E. faecium*. These results demonstrate that oxidized Crp4 has a more selective bactericidal activity against small intestinal bacteria. Thus, it is suggested that Crp4 plays a role in the regulation of intestinal microbiota by killing certain noncommensal species while retaining the viability of certain commensal bacteria. This concept was supported by the result that oxidized Crp4 selectively killed *S. enterica* serovar Typhimurium but showed no bactericidal activity against 2 species of com-

mensal bacteria, i.e. *L. casei* and *B. thetaiotaomicron*, when a mixture of bacterial species was exposed to oxidized Crp4. Oxidized Crp1 also showed selective bactericidal activities, suggesting that other members of Crps contribute to maintaining intestinal microbiota by selectively killing noncommensal bacteria. A recent study of intestinal microbiota in MMP-7-deficient mouse types that lacked the active form of Crps showed that a significantly higher percentage of Firmicutes and a significantly lower percentage of Bacteroides were detected in the small bowel of MMP-7-deficient mice compared to wild-type mice [33]. Since the total bacterial numbers in both types of mouse were not changed, it appears that Crps regulated the composition of the intestinal bacteria [33], consistent with our results that demonstrate the selective bactericidal activity of Crp4.

Some commensal bacteria showed a resistance to oxidized Crp4. To date, various bacterial resistance mechanisms against AMPs have been reported [34]. These include the proteolytic degradation of AMPs by microbial proteases [35], the binding of secreted bacterial proteins to AMPs to prevent AMPs from accessing the bacterial plasma membrane [36], the extrusion of AMPs from the bacterial cell by a multiple drug resistance exporter [37], and the modification of the bacterial cell membrane to reduce the net anionic charge, resulting in an attenuation of the affinity of AMPs to the surface membrane [38, 39]. A wide variety of microbicidal mechanisms, such as the permeabilization of bacterial cell membranes and the inhibition of DNA or protein synthesis, are presented for various AMPs [40]. In the case of Crp4, it has been shown that Crp4 permeabilized the phospholipid bilayer and that the activity was dependent on the membrane composition [41]. However, the precise bactericidal mechanisms of native Crp4 have yet to be fully elucidated and may be bacteria dependent. Therefore, the bacterial resistance mechanisms against Crp4 may also be varied and bacteria dependent. We detected a depolarization of the membrane potential in some noncommensal bacteria by Crp4. Further investigation of the bacterial factors that affect bactericidal activity is needed to uncover the bacterial resistance mechanisms against Crp4.

In this study, *Bifidobacterium* sp., *Lactobacillus* sp., *Bacteroides* sp., and *Enterococcus* sp. were used as types of small intestinal commensal bacteria. These bacteria have been reported to colonize mouse small intestine where Crp4 is present [42, 43]. Oxidized Crp4 did not show bactericidal activities against *B. bifidum*, *Lactobacillus* sp., *B. thetaiotaomicron*, or *B. fragilis*, while it did kill *B. longum*, *B. vulgatus*, and *Enterococcus* sp. *B. bifi-*

dum comprises the major portion of the intestinal microbiota in breast-fed infants [27]. *B. longum* is mainly found in adult intestine [28], and *L. acidophilus*, *L. casei*, *L. johnsonii*, *B. thetaiotaomicron*, *B. fragilis*, and *B. vulgatus* colonize the intestinal tract in both infants and adults [29, 30]. *Enterococcus* sp. are commensal bacteria that can cause opportunistic infection. It is speculated that the bacteria-dependent bactericidal activities of oxidized Crp4 against commensal bacteria reflect an in vivo regulatory role of Crp4 in tuning microbial homeostasis.

The bactericidal activities of r-Crp4 which contained no disulfide bond against small intestinal bacteria were analyzed. r-Crp4 had significantly greater bactericidal activities against *B. bifidum*, *B. breve*, *L. casei*, *L. johnsonii*, *B. fragilis*, *B. ovatus*, and *B. thetaiotaomicron* compared to oxidized Crp4. This result shows that the bactericidal activities of Crp4 against certain commensal bacteria were regulated by a function of its disulfide bonds, indicating a novel role of the conserved disulfide bonds of Crp4 in controlling bactericidal activities. r-Crp4 showed significantly greater depolarization activity than did oxidized Crp4 against the 3 commensal bacterial species tested. These results suggest that a disruption of membrane integration may account for part of the potent bactericidal activities of r-Crp4 relative to oxidized Crp4, especially with respect to certain commensals. Meanwhile, r-Crp4 and oxidized Crp4 showed equivalent bactericidal activities against 11 of 11 noncommensal bacteria and against 5 of 12 commensal bacteria. These results demonstrate that the bactericidal activities of Crp4 against these bacteria have no relationship with the presence of disulfide bonds. Previously, Maemoto et al. [25] showed that the bactericidal activity of Crp4 against noncommensal bacteria was independent of its disulfide array by using a disulfide-null mutant of Crp4. Our results show that the reduction of disulfide bonds has an effect against noncommensal bacteria compatible with the previous study. Thus, the regulatory effect of disulfide bonds depends on the bacterial species. r-Crp1 also showed the bacteria-dependent regulatory effects of disulfide bonds. It is speculated that this property of disulfide bonds may be applicable to other Crps. Native Crp4 consists of a triple-stranded antiparallel β -sheet [44], whereas the NMR spectroscopy of Cys-to-Ala-substituted Crp4 indicates the mutant peptide is disordered [25]. According to these structural data, the r-Crp4 used in this study may also have a random coil structure. In that case, it is suggested that the flexibility of r-Crp4 makes it bactericidal against the *B. bifidum*, *L. casei*, *L. johnsonii*, *B. thetaiotaomicron*, and *B. fragilis* that oxidized Crp4 did not kill completely.

In vitro degradation assays revealed that r-Crp4 was digested by MMP-7. Further, the bactericidal activities of r-Crp4 against small intestinal microbiota, except for *L. acidophilus*, were attenuated by this digestion. If r-Crp4 were to be secreted into the lumen of the small intestine, r-Crp4 would kill both noncommensal and commensal bacteria, resulting in the perturbation of small intestinal microbial homeostasis. Thus, it is considered that degradation and inactivation of r-Crp4 before secretion would prevent this perturbation. This suggests that the host has a management mechanism to avoid the release of aberrant Crp4 that is disadvantageous to the host.

Previously, it was reported that the proform of human Paneth cell α -defensin, i.e. HD5, was reduced in some patients with Crohn's disease [45]. The reduced pro-HD5 was degraded by trypsin, a processing enzyme of HD5, in vivo. This resulted in a diminished production of mature HD5 [45]. If Crp4 were to be reduced in vivo, the degradation of Crp4 by MMP-7 would lead to the dysfunction of innate immunity. Therefore, these results indicate that the protease resistance of α -defensins due to the disulfide bonds may contribute to the maintenance of intestinal innate immunity as well as the pathology of diseases such as inflammatory bowel disease.

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